Supplemental Figure Legends

**Supplemental Figure 1. ZO-1 localizes specifically to nephrocyte SDs.**
(A) Airy-scan imaging of WT nephrocytes stained with anti-ZO-1 antibody shows that ZO-1 is specifically localized to the curvilinear SDs that cover the surface of normal nephrocytes. (A') Cropped view of the region from (A) marked by the 1μm scale bar, which spans approximately four SDs. (B) TEM imaging confirms that SDs are spaced approximately 200-250nm apart.

**Supplemental Figure 2. Dlg1, but not Dlg5, is required for correct localization of ZO-1 and Nephrin.**
(A) Control nephrocyte has normal surface enrichment of ZO-1 (green). (B) Animals heterozygous for both Sns-Gal4 and Dlg RNAi transgenes have significantly reduced surface ZO-1 (red dashed line outlines cell boundaries). (C) This phenotype is significantly increased in animals homozygous for both the Gal4 and RNAi transgenes. (D) Quantification of ZO-1 localization in these animals. (E) A different Dlg RNAi (#33620) line causes similar defects as those observed using the primary RNAi line of this study (#36771). (F) Nephrocytes from flies harboring a temperature-sensitive mutation in *dlg* and raised at the restrictive temperature of 29C show severe defects in ZO-1 localization. (G) KD of fly Dlg5 has no appreciable effect on ZO-1 localization.
(H) Similar to ZO-1 and Neph1 (see Fig.2) the fly Nephrin homolog localizes to the surface of nephrocytes. (I) In Dlg KD cells, Nephrin is also mislocalized from the cell surface and into internal aggregations. Scale bars=10μm.

**Supplemental Figure 3. Cytoplasmic aggregates of Dlg and Lgl do not colocalize with the ER, Golgi, or Par3.**
(A) Lgl (red) and Dlg (blue) can colocalize at cytoplasmic "clouds" in control nephrocytes, but this does not overlap with a Golgi:eYFP marker (green). (B) Similarly, the cytoplasmic aggregations of Lgl (red) and Dlg (blue) does not colocalize with an ER:eYFP marker (green). (C) We also did not observe significant enrichment of Par3 (red) at Dlg clouds (green). Scale bars=10μm.

**Supplemental Figure 4. Dlg and Par-1 promote normal Crb localization and protein levels.**
(A) In control nephrocytes (Dot-Gal4 alone), Crb is enriched near the cell periphery. (B) In Dlg KD cells, Crb is mislocalized into cytoplasmic aggregates of varying sizes, including several large puncta (green arrows). (C) In Par-1 KD cells, Crb is also mislocalized, however it is found in many small puncta spread more evenly distributed in size. (D) Quantification of Crb fluorescence intensity reveals a reduction in mean levels of Crb in both Dlg KD and Par-1 KD cells. This effect appears more severe in Dlg KD compared to Par-1 KD. Scale bars=10μm.

**Supplemental Figure 5. Mislocalized ZO-1 in Dlg KD cells does not accumulate in the ER or Golgi, but their organization is perturbed by loss of Dlg, Nephrin, or Neph1.**
(A) In WT nephrocytes, the ER marker Sec61B:tdTomato (red) appears as a homogenous cloud surrounding the nuclei in the center of the cell, while the Golgi marker Golgin84 (green) reveals punctate structures interspersed along the outer edge of the ER. (B) In Dlg KD cells, mislocalized ZO-1 (red) does not colocalize with the Golgi (green), however, the Golgi organization is severely disrupted in these cells; compare B' to A'. (C) While mislocalized ZO-1 (green) is also not present in the ER, the ER itself is disrupted in Dlg KD cells, appearing fragmented through the cytoplasm with uneven concentrations of the ER marker Sec61B (red); compare C" to A". (D) Aberrant Golgi

organization was also observed in cells depleted of Nephrin. Similarly, the ER appears abnormal in cells depleted of Nephrin (E) or Neph1 (F). Scale bars=10μm.

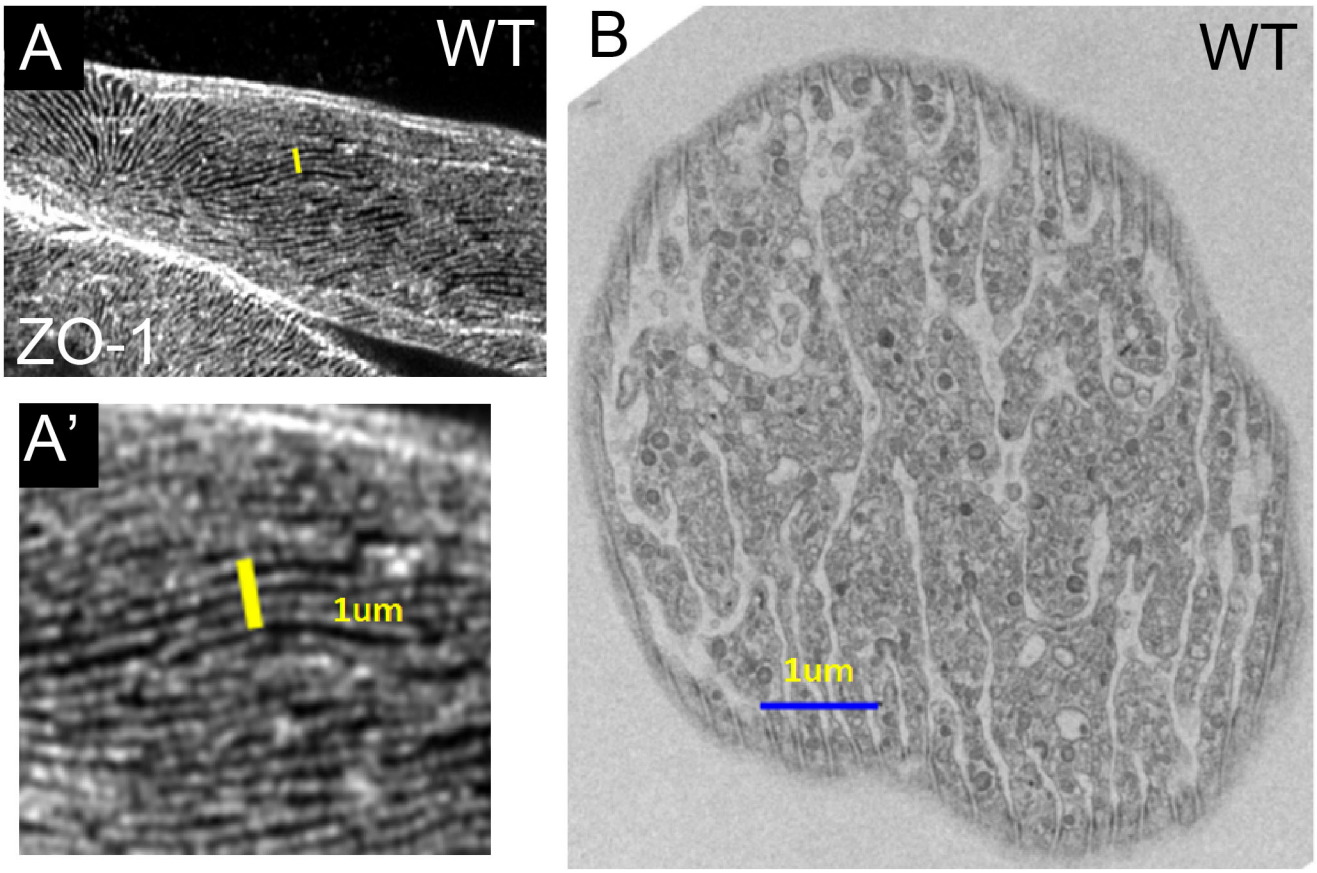
**Supplemental Figure 6. In Dlg KD nephrocytes, mislocalized ZO-1 does not colocalize with Rab7 or Rab11, despite their aberrant subcellular distributions.**
(A) A subcortical slice of WT nephrocytes shows numerous Rab7-positive vesicles and larger circular structures (yellow arrows), which are likely late endosomes, lysosomes, and α-vacuoles (Rab7 in green; ZO-1 in red)[65]. (B) A medial cross-section of a WT cell indicates that the majority of Rab7-vesicles are located in the subcortical region of the cell. (C-D) In Dlg KD cells, subcortical and medial sections reveal an even distribution of Rab7 structures throughout the cell, and lack the larger circular Rab7 structures. Note that the mislocalized ZO-1 protein does not colocalize with Rab7 in Dlg KD cells. (E) WT nephrocytes show cortical enrichment of Rab11 vesicles (green), just interior to the ZO-1 positive SD region (red). (F) In Dlg KD cells, Rab11 localization is diffuse through the cytoplasm and does not colocalize with internally mislocalized ZO-1 aggregations. Scale bars=10μm.

**Supplemental Figure 7. Endocytic markers Rab7 and Rab11 are not specifically enriched in the aberrant ZO-1 aggregations caused by Par-1 KD.**
(A) The "cloud" of mislocalized SD protein ZO-1 (red) that is typical of Par-1 KD contains some Rab7 structures (green). However, in contrast to Rab5 (see Fig.5D,E), Rab7 structures do not preferentially aggregate at these sites—they appear evenly distributed throughout the cytoplasm.
(B) Similarly, although Rab11 (green) can be detected in the ZO-1 aggregations in Par-1 KD, this does not appear to be a specific recruitment to that site, as seen with Rab5. Note that the more uniform distribution of Rab7 and Rab11 in Par-1 KD is qualitatively different from WT nephrocytes (compare to Suppl.Fig.6B',E'). Scale bars=10μm.

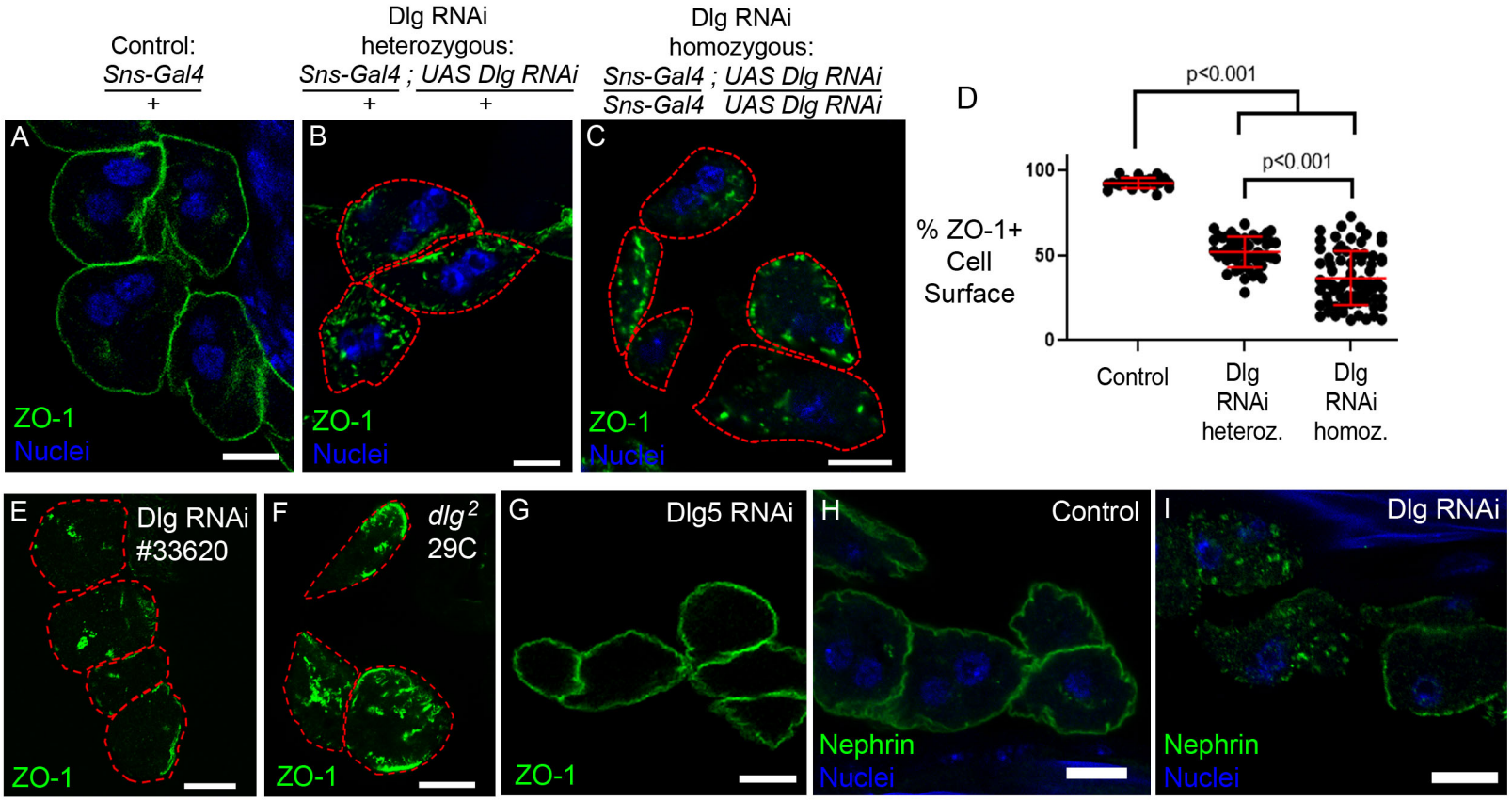
**Supplemental Figure 8. In nephrocytes depleted of Dlg or Par-1, mislocalized Nephrin and Neph1 are adjacent to Rab5 structures, while only loosely associated with Rab11 and Rab7.**
(A) In Dlg KD cells, Nephrin is mislocalized to cytoplasmic aggregates (yellow arrows), which are intermixed with mislocalized Rab5. (B) Similarly, in Par-1 KD cells, Nephrin puncta are tightly associated with dense accumulations of Rab5 vesicles, thought they do not appear to colocalize. (C) Dlg KD causes a uniform disruption of Rab11 distribution, which can contain mislocalized Neph1, though again there is no colocalization or specific association of the two proteins. (D) Similar results were observed in Par-1 KD. (E) In Dlg KD, mislocalized Rab7 and Neph1 are both mislocalized, but not significantly associated with one another.

**Supplemental Figure 9. Scrib is required for normal rates of endocytosis.**
(A) Wildtype nephrocytes readily internalize 10kD fluorescent dextran. (B) Nephrocytes null mutant for *scrib* have significantly reduced dextran uptake. (C) Quantification of dextran signal shows significantly reduced fluorescence intensity in *scrib* mutant cells. Scale bars=10μm.

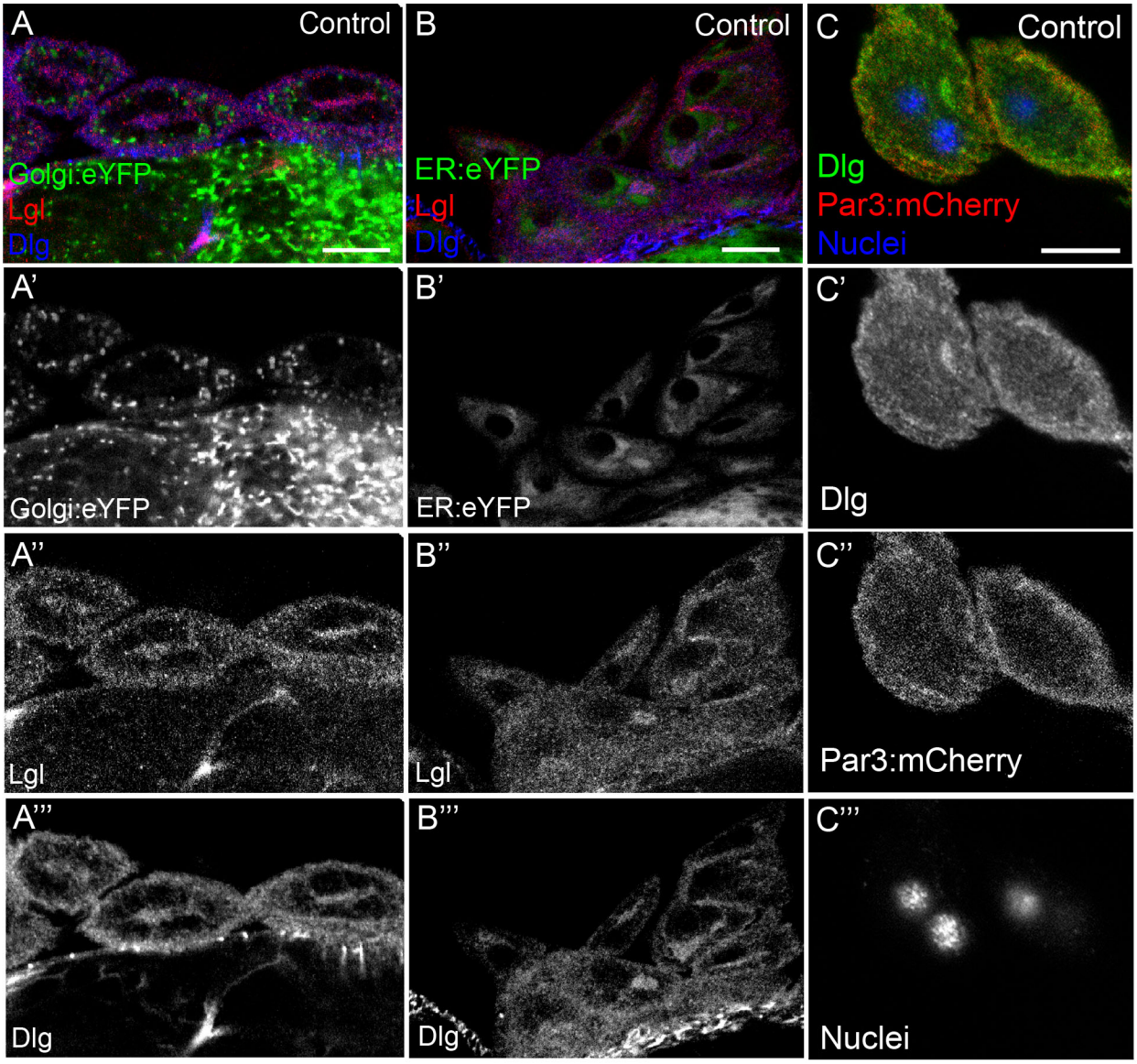
**Suppl.Fig.1**



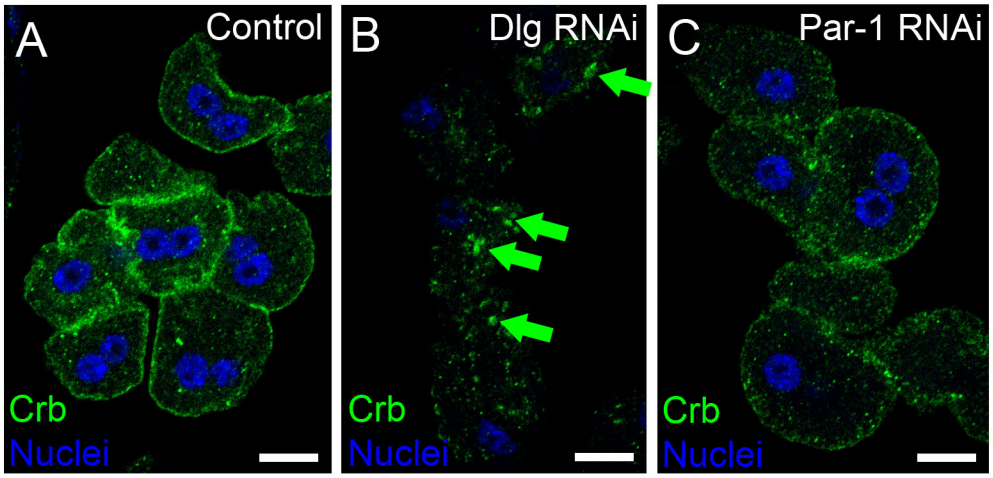
Suppl.Fig.2

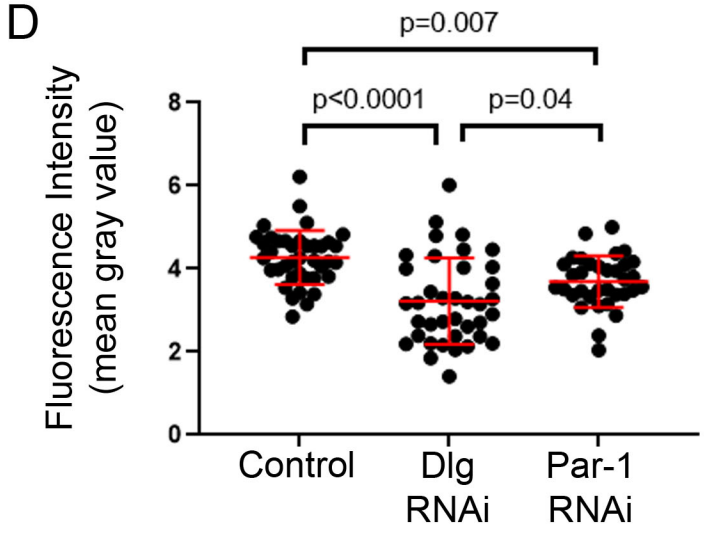

Control:
Sns-Gal4
+
Dlg RNAi
heterozygous:
Sns-Gal4 ; UAS Dlg RNAi
+ +
Dlg RNAi
homozygous:
Sns-Gal4 ; UAS Dlg RNAi
Sns-Gal4 UAS Dlg RNAi
A
ZO-1
Nuclei
B
ZO-1
Nuclei
C
ZO-1
Nuclei
D
p<0.001
p<0.001
% ZO-1+ Cell Surface
100
50
0
Control
Dlg RNAi heteroz.
Dlg RNAi homoz.
E
Dlg RNAi #33620
ZO-1
F
dlg2 29C
ZO-1
G
Dlg5 RNAi
ZO-1
H
Control
Nephrin
Nuclei
I
Dlg RNAi
Nephrin
Nuclei

Suppl.Fig.3

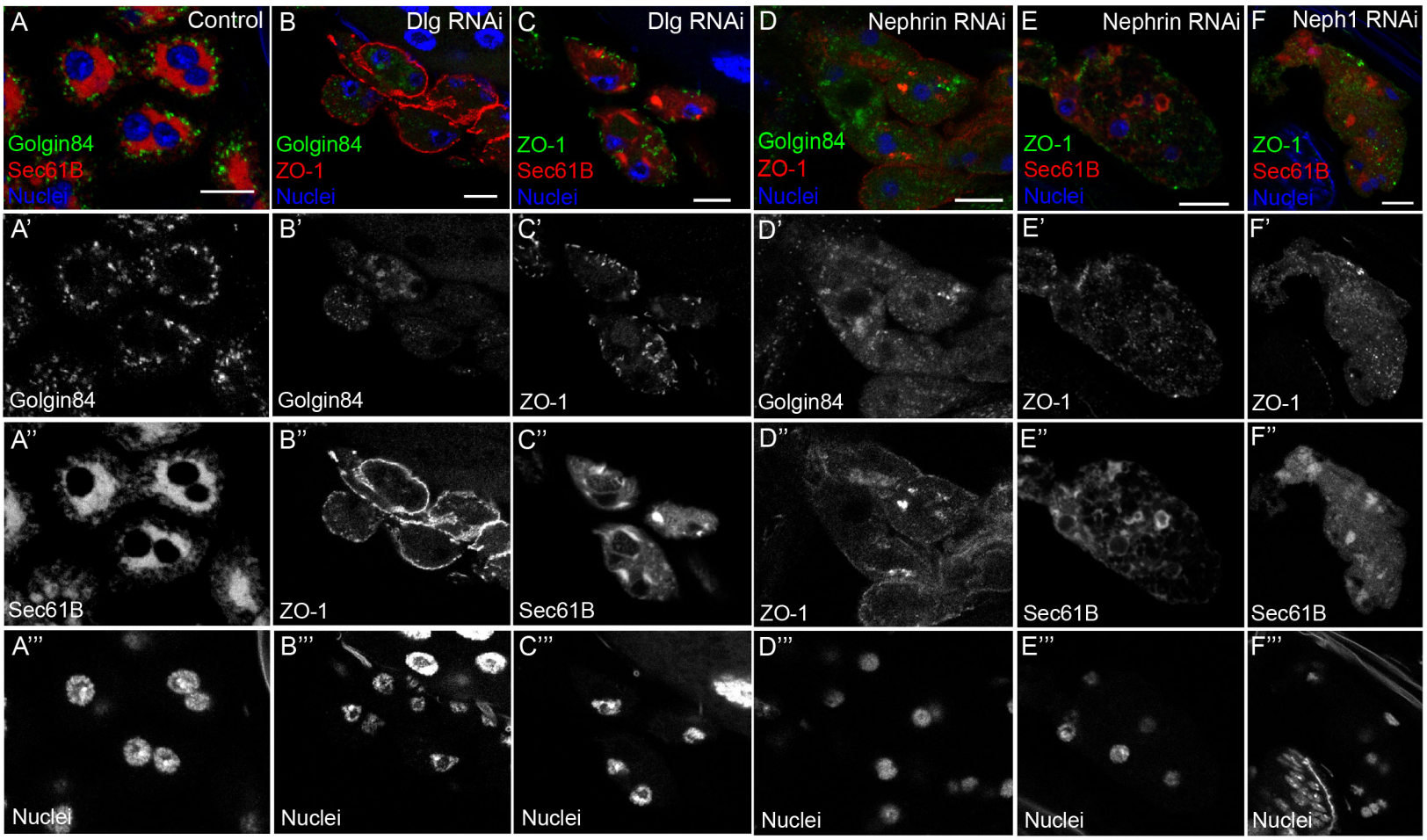

A
Control
Golgi:eYFP
Lgl
Dlg
B
Control
ER:eYFP
Lgl
Dlg
C
Control
Dlg
Par3:mCherry
Nuclei
A'
Golgi:eYFP
B'
ER:eYFP
C'
Dlg
A''
Lgl
B''
Lgl
C''
Par3:mCherry
A'''
Dlg
B'''
Dlg
C'''
Nuclei

Suppl.Fig.4

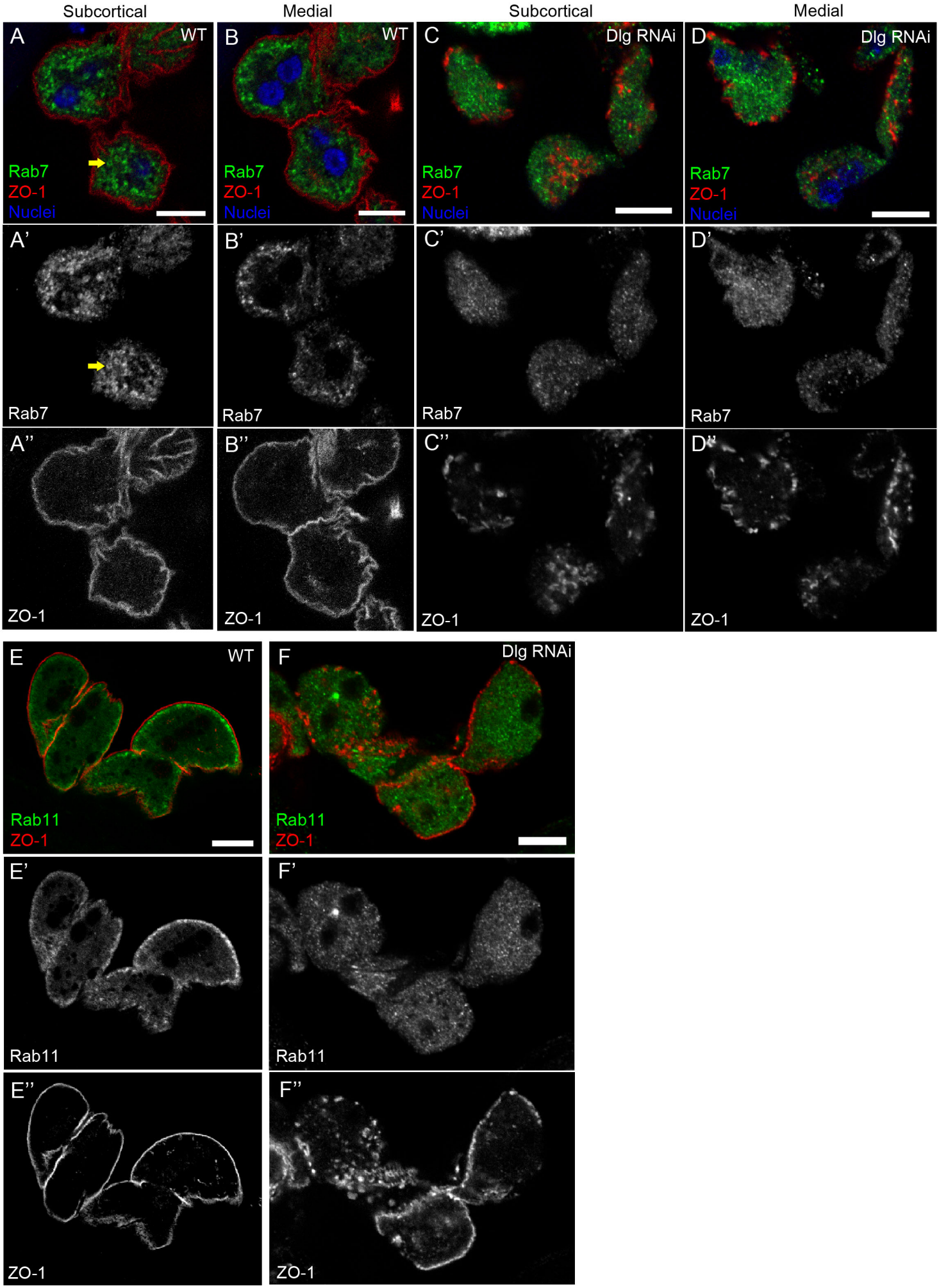

A
Control
Crb
Nuclei
B
Dlg RNAi
Crb
Nuclei
C
Par-1 RNAi
Crb
Nuclei


D
p=0.007
p<0.0001
p=0.04
Fluorescence Intensity
(mean gray value)
8
6
4
2
0
Control
Dlg
RNAi
Par-1
RNAi

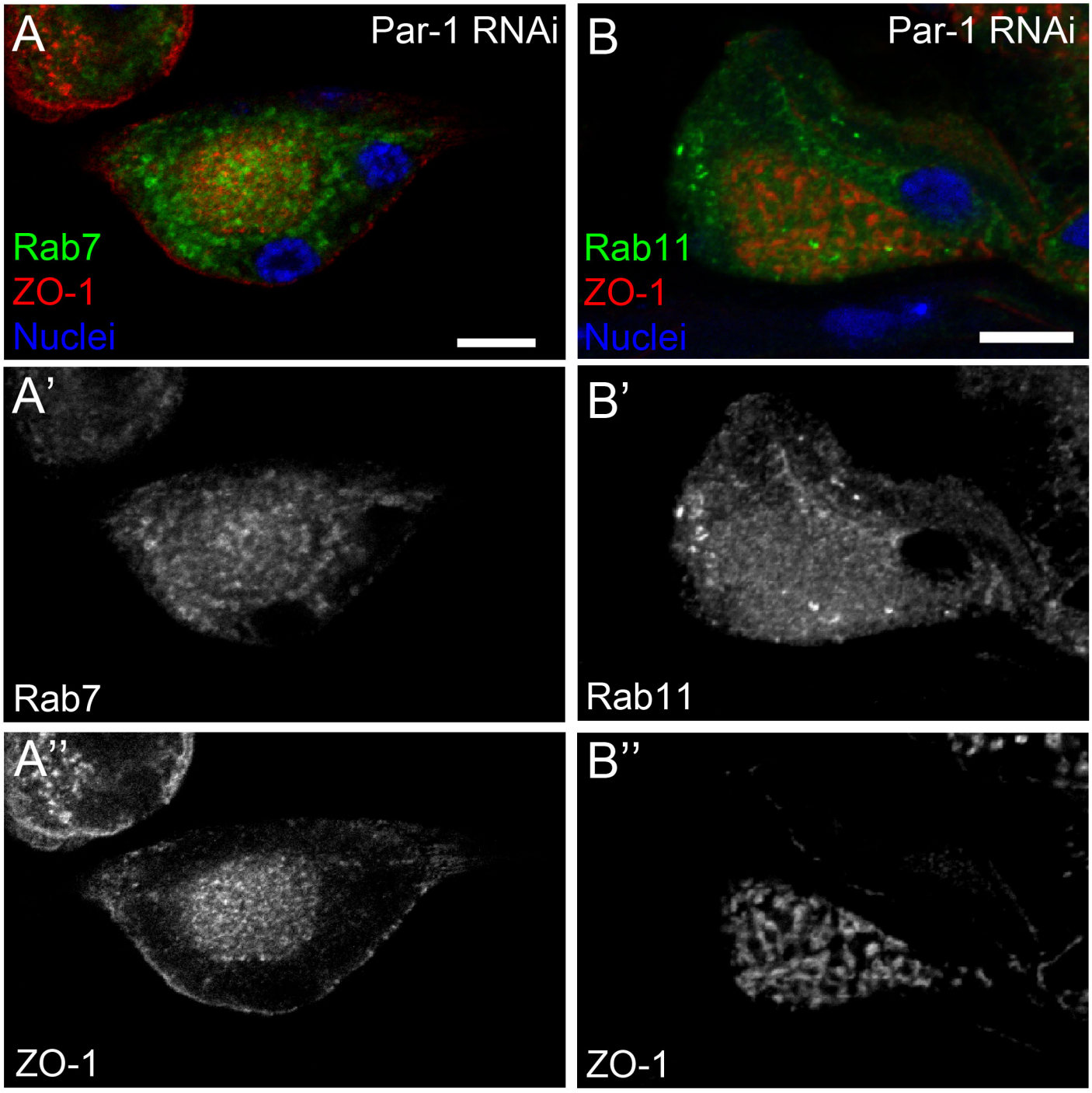
**Suppl.Fig.5**


A
Control
Golgin84
Sec61B
Nuclei
B
Dlg RNAi
Golgin84
ZO-1
Nuclei
C
Dlg RNAi
ZO-1
Sec61B
Nuclei
D
Nephrin RNAi
Golgin84
ZO-1
Nuclei
E
Nephrin RNAi
ZO-1
Sec61B
Nuclei
F
Neph1 RNAi
ZO-1
Sec61B
Nuclei
A'
Golgin84
B'
Golgin84
C'
ZO-1
D'
Golgin84
E'
ZO-1
F'
ZO-1
A''
Sec61B
B''
ZO-1
C''
Sec61B
D''
ZO-1
E''
Sec61B
F''
Sec61B
A'''
Nuclei
B'''
Nuclei
C'''
Nuclei
D'''
Nuclei
E'''
Nuclei
F'''
Nuclei

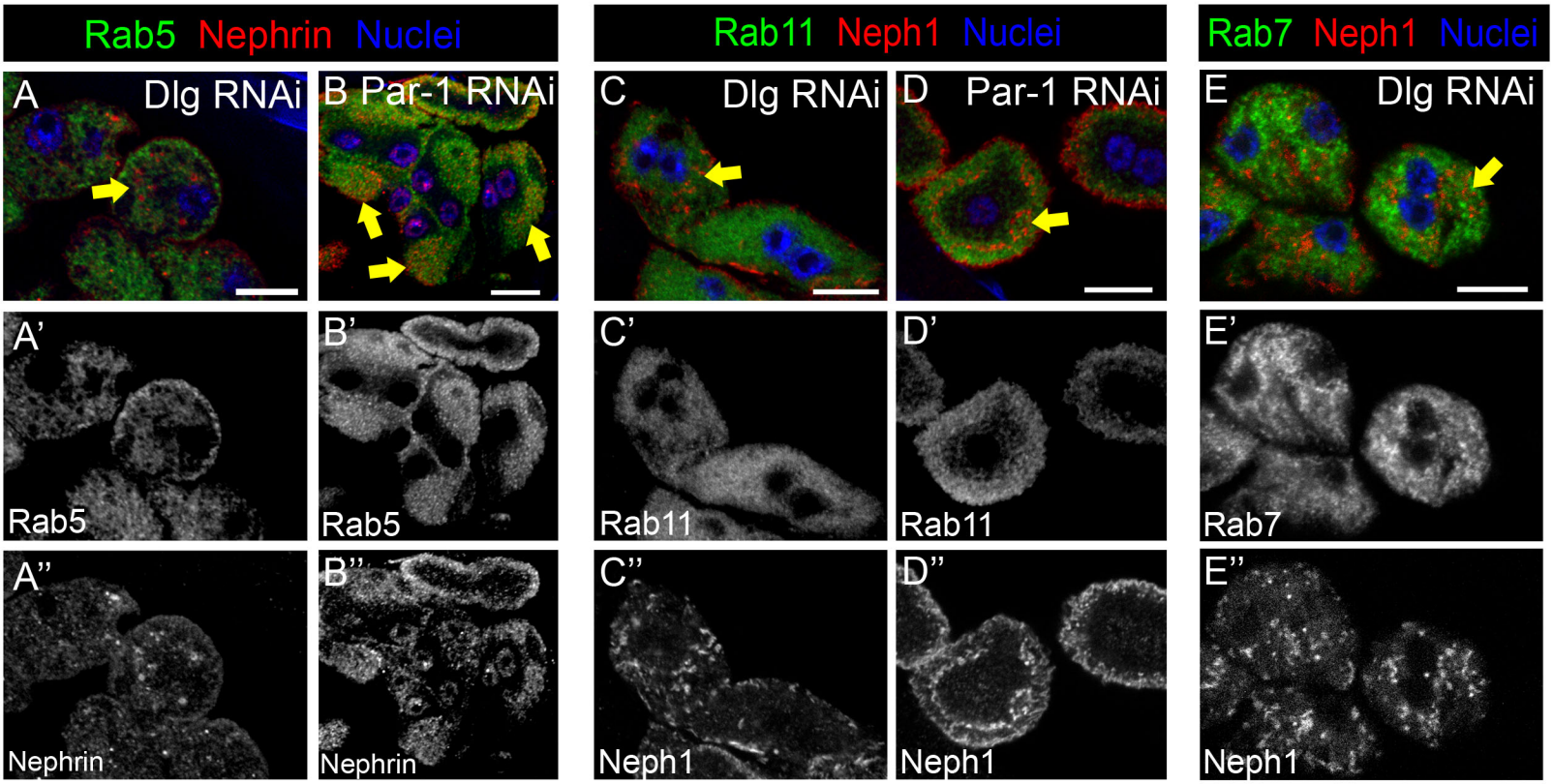
**Suppl.Fig.6**


Subcortical
Medial
Subcortical
Medial
A
WT
Rab7
ZO-1
Nuclei
B
WT
Rab7
ZO-1
Nuclei
C
Dlg RNAi
Rab7
ZO-1
Nuclei
D
Dlg RNAi
Rab7
ZO-1
Nuclei
A'
Rab7
B'
Rab7
C'
Rab7
D'
Rab7
A''
ZO-1
B''
ZO-1
C''
ZO-1
D''
ZO-1
E
WT
Rab11
ZO-1
F
Dlg RNAi
Rab11
ZO-1
E'
Rab11
F'
Rab11
E''
ZO-1
F''
ZO-1

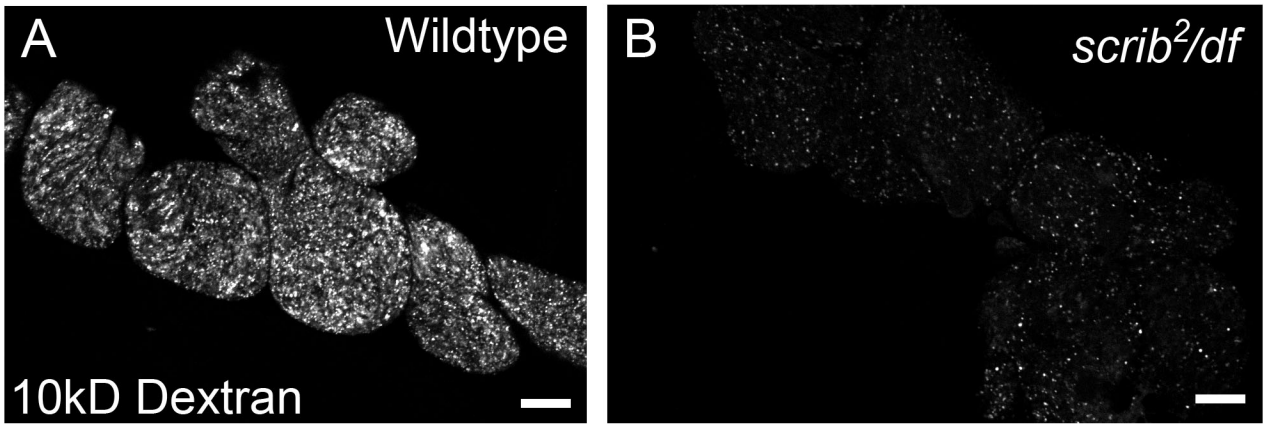
**Suppl.Fig.7**

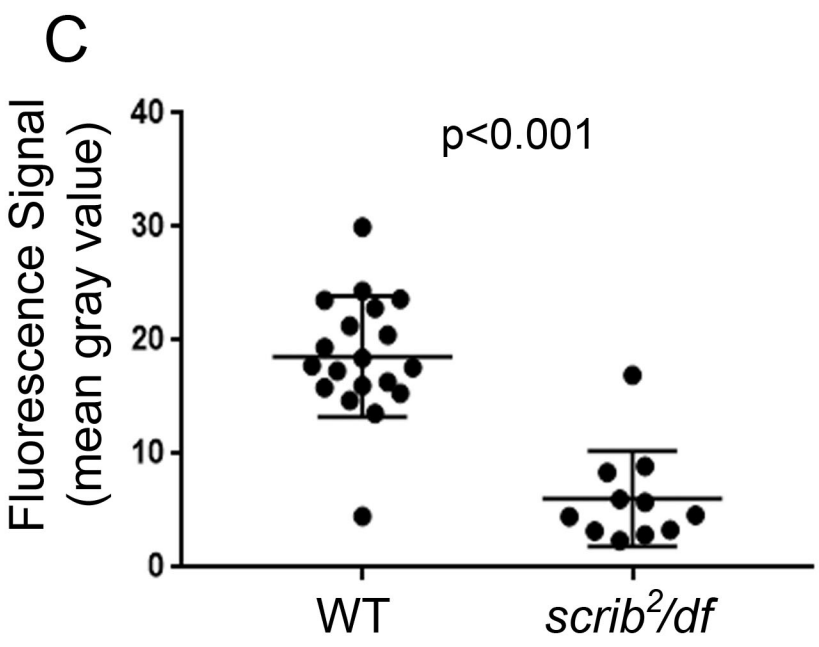

A
Par-1 RNAi
Rab7
ZO-1
Nuclei
B
Par-1 RNAi
Rab11
ZO-1
Nuclei
A'
Rab7
B'
Rab11
A''
ZO-1
B''
ZO-1

Suppl.Fig.8


Rab5 Nephrin Nuclei
Rab11 Neph1 Nuclei
Rab7 Neph1 Nuclei
A Dlg RNAi
B Par-1 RNAi
C Dlg RNAi
D Par-1 RNAi
E Dlg RNAi
A'
B'
C'
D'
E'
Rab5
Rab5
Rab11
Rab11
Rab7
A''
B''
C''
D''
E''
Nephrin
Nephrin
Neph1
Neph1
Neph1

Suppl.Fig.9


A
Wildtype
10kD Dextran
B
scrib2/df


C
Fluorescence Signal
(mean gray value)
40
30
20
10
0
p<0.001
WT
scrib2/df

## Suppl Table 1. List of antibodies used in this study

| Antigen | Antibody info/clone | Animal | Dilution | Source |
|---|---|---|---|---|
| ZO-1/Pyd | PYD2 | Mouse | 1:500 | DSHB |
| Dlg | 4F3 | Mouse | 1:50 | DSHB |
| Golgin84 | 12-1 | Mouse | 1:40 | DSHB |
| Lgl | 98260 | Rabbit | 1:200 | Santa Cruz |
| Scrib | | Guinea Pig | 1:500 | David Bilder |
| Par-1 | | Guinea Pig | 1:800 | Denise Montell |
| Crb | ex3 | Rat | 1:1000 | Elixabeth Knust |
| Rab5 | | Guinea Pig | 1:2000 | Akira Nakamura |
| Rab7 | | Rabbit | 1:1000 | Akira Nakamura |
| Rab11 | | Rabbit | 1:1000 | Akira Nakamura |
| Nephrin/Sns | | Rabbit | 1:1000 | Susan Abmayr |
| Neph1/Duf | | Guinea Pig | 1:1000 | Susan Abmayr |

DSHB = Developmental Studies Hybridoma Bank